Acid sphingomyelinase is involved in CEACAM receptor-mediated phagocytosis of *Neisseria gonorrhoeae*

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Received 16 May 2000; revised 29 June 2000; accepted 30 June 2000

Edited by Felix Wieland

Abstract The interaction with human phagocytes is a hallmark of symptomatic *Neisseria gonorrhoeae* infections. Gonococcal outer membrane proteins of the Opa family induce the opsonin-independent uptake of the bacteria that relies on CEACAM receptors and an active signaling machinery of the phagocyte. Here, we show that CEACAM receptor-mediated phagocytosis of Opa_{52}-expressing *N. gonorrhoeae* into human cells results in a rapid activation of the acid sphingomyelinase. Inhibition of this enzyme by imipramine or SR33557 abolishes opsonin-independent internalization without affecting bacterial adherence. Reconstitution of ceramide, the product of acid sphingomyelinase activity, in imipramine- or SR33557-treated cells restores internalization of the bacteria. Furthermore, we demonstrate that CEACAM receptor-initiated stimulation of other signalling molecules, in particular Src-like tyrosine kinases and Jun N-terminal kinases, requires acid sphingomyelinase. These studies provide evidence for a crucial role of the acid sphingomyelinase for CEACAM receptor-initiated signalling events and internalization of Opa_{52}-expressing *N. gonorrhoeae* into human neutrophils.

Key words: Gonococcus; Uptake; Sphingolipid; Src family kinase; Signalling

1. Introduction

Neisseria gonorrhoeae, the causative agent of the venereal disease gonorrhea, is a highly adapted pathogen with only a single natural host, man. During the course of symptomatic infections, these bacteria are capable of interacting with a variety of human cell types including epithelial cells and phagocytes [1]. To accomplish the intimate attachment to different types of host cells, gonococci express a family of phase variable outer membrane proteins, the so-called opacity-associated or Opa proteins. Several studies have established that particular Opa variants (Opa_{50}, OpaA) mediate the binding of the microorganisms to heparan sulfate proteoglycan receptors on epithelial cells leading to the uptake of the bacteria by non-phagocytic cells [2,3]. Internalization of these gonococcal strains requires an activation of the cellular acid sphingomyelinase [4], an enzyme which is able to induce the formation of endocytic vesicles [5].

On the other hand, a distinct set of Opa proteins has been shown to associate with members of the CEACAM receptor family (formerly CD66 receptor family) on epithelial cells and phagocytes [6–8]. In particular, Opa_{52}, a representative of this group of Opa proteins, recognizes CEACAM1 (CD66a, biliary glycoprotein, BGP), CEACAM5 (CD66c, non-specific crossreacting antigen 90, NCA90) and CEACAM3 (CD66d, CGM1) receptors, that are expressed on human neutrophils [9]. Binding of Opa_{52}-expressing bacteria to human phagocytic cells triggers intracellular tyrosine phosphorylation of multiple substrates that is dependent on the CEACAM receptor-mediated activation of the Src-like tyrosine kinases Hck and Fgr and the down-modulation of SHP-1 tyrosine phosphatase activity [10,11]. In addition, Opa_{52}-mediated interactions lead to activation of the small GTPase Rac and the stress-activated protein kinase JNK [10].

Since the signalling events triggered by Opa_{50}-expressing gonococci in epithelial cells and the engagement of CEACAM receptors by Opa_{52}-expressing bacteria ultimately lead to the efficient opsonin-independent uptake of the microorganisms, we tested whether both pathways are interconnected. In the present study we provide evidence for a requirement of the acid sphingomyelinase for opsonin-independent internalization of *N. gonorrhoeae* into human phagocytes. Infection of the cells with Opa_{52}-expressing *N. gonorrhoeae* results in a rapid activation of the acid sphingomyelinase, Src-like tyrosine kinases and Jun-N-terminal kinases (JNK). Experiments with pharmacological inhibitors of the acid sphingomyelinase as well as of Src-like tyrosine kinases indicate that the acid sphingomyelinase functions upstream of Src-like tyrosine kinases and JNK activation.

2. Materials and methods

2.1. Cells, bacteria and infection

The human myelomonocytic cell line JOSK-M was cultured and differentiated in vitro as described [12]. As indicated, cells were pre-incubated with imipramine (50 μM) for 15 min, SR33557 (10 μM) for 1 min or lavendustin (10 μM) for 30 min prior to infection. C_{16} ceramide (50 nM) dissolved in 0.01% octyl-glucopyranoside was added to imipramine or SR33557-treated or untreated control cells 5 min prior to infection. The gonococcal strains used in the present work are exclusively derived from *N. gonorrhoeae* MS11 [13]. N280, a piliated strain exhibiting the transparent phenotype (Opa^-, P^+), as well as the non-piliated strain N309 expressing a phagocyte-specific Opa protein (Opa_{52}, P^-) have been previously described [10]. Commensal *Neisseria cinerea* (N340) was originally obtained from U. Berger, Heidelberg,

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0014-5793/00/$20.00
PII: S0014-5793(00)01851-2

Germany. All *Neisseria* were grown on GC-agar (Life Science Technologies) supplemented with vitamins and antibiotics, where appropriate, at 37°C in 5% CO_2 and subcultured daily. Recombinant *Escherichia coli* DH5α producing Opa_{52} (H1907) and DH5α harboring the empty expression vector pTrc99A (H1887) were maintained on LB-plates supplemented with 100 μg/ml ampicillin. For infection, plate grown bacteria were suspended in RPMI1640, washed by centrifugation at 4000 rpm for 5 min in a microcentrifuge and resuspended. The optical density at 550 nm (gonococci) or 600 nm (*E. coli*) was determined in a DR2000 spectrophotometer (Hach, Coveland, CO, USA) and bacteria were added to differentiated JOSK-M cells in RPMI1640 supplemented with 5% heat inactivated FCS at a ratio of 50 bacteria per cell at 37°C to start the infection. After the indicated time, cells were pelleted by centrifugation at $250\times g$ for 2 min at 4°C (for immunoprecipitation) or the cells were washed two times with PBS for 5 min at $100\times g$ in a microcentrifuge and then centrifuged on glass coverslips for 5 min at $50\times g$ (for immunofluorescence staining). Internalization was also determined by crystal violet staining. To this end, cells were fixed for 15 min in 1% paraformaldehyde in PBS, washed and stained for 12 h at 4°C in 0.07% crystal violet. Intracellular bacteria were microscopically identified and counted from at least 100 cells.

2.2. Acid sphingomyelinase-activity

Infected or uninfected cells were lysed in 50 mM Tris (pH 7.4), 10 mM bacitracin, 1 mM benzamidine, 10 mM Na_3VO_4, 10 μg/ml of each aprotinin and leupeptin (A/L), 0.1 mg/ml soybean trypsin inhibitor and 0.2% Triton X-100 (lysis buffer), sonicated three times for 10 s each and centrifuged at $600\times g$ for 5 min. All samples were normalized by labelling of the mammalian cells with [^{3}H]thymidine for 24 h prior to infection. An aliquot of the lysates was used to normalize for equal amount of cells. The labelling method for normalization was employed to avoid interference with proteins from the bacteria. An equal amount of 50 mM Tris (pH 7.4), 3% NP40, 1% Triton X-100, 10 mM Na_3VO_4, 100 μg/ml each A/L (lysis buffer) was added to the supernatants and the acid sphingomyelinase was immunoprecipitated for 5 h at 4°C using a goat-anti-acid sphingomyelinase antiserum immobilized on protein A/G agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoprecipitates were washed three times each in lysis buffer and 50 mM sodium acetate (pH 5.0), 0.2% Triton X-100, 1 mM Na_3VO_4 and 10 μg/ml each A/L and then incubated with [^{14}C]sphingomyelin (100 nCi/sample, 54.5 mCi/mmol; NEN-DuPont) in 250 mM sodium acetate (pH 5.0), 1.3 mM EDTA, 0.05% NP40 (assay buffer) at 37°C for 30 min. Dried [^{14}C]sphingomyelin was solubilized by 10 min bath sonication in assay buffer. Samples were finally extracted with $CHCl_3$:CH_3OH (2:1, v/v) and H_2O. The upper phase was collected and the release of [^{14}C]phosphorylcholine was determined by liquid scintillation counting (LSC). Unspecific immunoprecipitates were performed with an irrelevant antibody. Those samples display a low background of 80–100 cpm compared to 2000–4000 cpm in the specific samples, which is probably due to some contamination during the extraction procedure.

2.3. Immunofluorescence microscopy

Infected cells were centrifuged onto glass coverslips in 24-well plates (Nunc, Roskilde, Denmark) and fixed for 20 min in 3% paraformaldehyde in PBS at room temperature. After three washes with PBS, cells were permeabilized for 5 min in PBS supplemented with 10% fetal calf serum and 0.2% saponin (blocking buffer). Suitable dilutions of polyclonal rabbit-anti-*N. gonorrhoeae* MS11 (AK92) and monoclonal mouse-anti-human lysosome-associated membrane protein 2 (h-lamp-2; clone H4B4; DSHB, University of Iowa, IA, USA) in blocking buffer were applied to the samples for 1 h at room temperature. Cells were washed twice with PBS, blocked again for 5 min and incubated with FITC-conjugated goat-anti-rabbit and Texas Red-conjugated goat-anti-mouse-antibodies (Dianova, Hamburg, Germany) for 45 min at room temperature. After three washes, coverslips were mounted in glycerol medium, sealed with nail polish, and viewed with a Leica TCS 4D confocal laser scanning microscope equipped with an argon–krypton mixed gas laser (Leica Lasertechnik, Heidelberg, Germany). Images were taken serially using appropriate excitation and emission filters for the fluorescent dyes. The corresponding images were digitally processed with Photoshop 4.0 (Adobe Systems, Mountain View, CA, USA) and merged to yield pseudocolored RGB pictures. Intracellular bacteria were detected by their co-localization with the phagosomal/lysosomal membrane protein h-lamp-2 and the percentage of infected cells was determined by counting at least 200 cells in each of three independent infections.

2.4. Src-like tyrosine kinase-assays

The activity of Src-like tyrosine kinases was determined by in vitro kinase autophosphorylation assays. To this end, cells were infected for the indicated time or left uninfected, lysed in 25 mM HEPES (pH 7.4), 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 125 mM NaCl, 10 mM each NaF, Na_3VO_4 and sodium pyrophosphate and 10 μg/ml of each aprotinin and leupeptin (A/L) (RIPA buffer), centrifuged and kinases Hck or Fgr, respectively, were immunoprecipitated from the supernatants using affinity purified rabbit anti-Hck or anti-Fgr antibodies (Santa Cruz Biotechnology). Immunoprecipitates were incubated at 4°C for 4 h, washed three times in RIPA buffer and three times in Src-kinase buffer (25 mM HEPES (pH 7.0), 150 mM NaCl, 10 mM $MnCl_2$, 1 mM Na_3VO_4, 5 mM DTT and 0.5% NP-40). Samples were resuspended in 40 μl Src-kinase buffer. The reaction was initiated by addition of 10 μCi [γ-^{32}P]ATP (NEN/Du-Pont, Bad Homburg, Germany; 3000 Ci/mmol) and ATP (10 μM) in Src-kinase buffer. The samples were incubated at 30°C for 20 min, the reaction was stopped with 8 μl of reducing 5×SDS sample buffer and SDS–PAGE was performed followed by autoradiography. An aliquot of the immunoprecipitates was analyzed by Western blotting for the amount of kinase in the immunoprecipitates.

2.5. JNK-1 activity

To measure the activity of JNK-1, cells were lysed in RIPA-buffer and the lysates were cleared by centrifugation at $25\,000\times g$ for 20 min. JNK-1 was immunoprecipitated from the supernatant at 4°C for 4 h using affinity purified rabbit anti-human JNK-1 antibodies (Santa Cruz Biotechnology). Immunocomplexes were immobilized on protein A/G for additional 60 min at 4°C, washed twice in RIPA-buffer, twice in 20 mM HEPES, 132 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.7 mM $MgCl_2$, 0.8 mM $MgSO_4$, 1% NP-40, 2 mM Na_3VO_4, once in 100 mM Tris (pH 7.5), 0.5 M LiCl and finally twice in JNK kinase buffer (12.5 mM MOPS (pH 7.5), 12.5 mM β-glycerophosphate, 0.5 mM EGTA, 7.5 mM $MgCl_2$, 0.5 mM NaF, 0.5 mM Na_3VO_4). After washing, the immunoprecipitates were resuspended in JNK-kinase buffer supplemented with 10 μCi [γ-^{32}P]ATP (6000 Ci/mmol, NEN/DuPont), 10 μM ATP and 1 mg/ml GST-c-jun (amino acids 1–79). The samples were incubated at 30°C for 15 min, stopped by addition of 5 μl boiling 5× reducing SDS-sample buffer. Samples were separated by 10% SDS–PAGE and electrophoretically transferred to PVDF membranes. Following autoradiography, the membranes were analyzed for equal amounts of immunoprecipitated kinase by probing with polyclonal JNK-1 antibodies.

3. Results

The present study aimed to define the interplay between the acid sphingomyelinase and Src-like tyrosine kinases, both have been previously shown to be crucial for the internalization of *N. gonorrhoeae* by human epithelial cells or Opa_{52}-expressing gonococci by phagocytic cells, respectively [4,10].

In order to investigate the role of the acid sphingomyelinase and Src-like tyrosine kinases in opsonin-independent phagocytosis, we infected in vitro differentiated human phagocytic cells with *N. gonorrhoeae* and determined the activity of the acid sphingomyelinase following immunoprecipitation of the enzyme (Fig. 1). The infection of JOSK-M cells with the Opa_{52}-expressing, non-piliated strain N309 resulted in a very rapid, approximately 1.5-fold activation of the acid sphingomyelinase. Acid sphingomyelinase activity in response to N309 peaked already 15 min after infection and declined to a baseline level within the following 45 min. In contrast, infection with the non-opaque, piliated strain N280 had no effect on cellular acid sphingomyelinase activity (Fig. 1). Likewise, infection with non-pathogenic *N. cinerea* (N340) did not stimulate this enzyme. Since activation of the acid sphingo-

myelinase was restricted to *N. gonorrhoeae* expressing Opa_{52}, we wondered whether expression of this Opa protein is sufficient to induce acid sphingomyelinase in target cells. Therefore, JOSK-M cells were infected with a strain of *E. coli* expressing Opa_{52} (H1907) or a control strain (H1887). Only Opa_{52}-expressing bacteria enhanced acid sphingomyelinase activity, whereas the control strain did not lead to a stimulation (Fig. 1).

The rapid activation of the acid sphingomyelinase suggest that stimulation of this enzyme plays a role in the early phase of CEACAM receptor signalling. To gain some insight into the potential role of the acid sphingomyelinase for the uptake process we tested the effect of two acid sphingomyelinase inhibitors, imipramine [14] and SR33557 [15], on *N. gonorrhoeae* N309 internalization by JOSK-M cells. Acid sphingomyelinase was almost completely inhibited by 15 min pre-incubation of the cells with 50 μM imipramine previously shown to trigger a degradation of acid sphingomyelinase [14]. SR33557 (10 μM) blocked acid sphingomyelinase activity within 30–60 s after application without significant degradation at the 75- or 72-kDa isoforms of ASM (not shown).

Sphingomyelinase inhibition was determined by immunoprecipitation of the enzyme and measurement of the enzyme activity in the precipitates revealed an approximately 80–90% inhibition of sphingomyelinase activity by 50 μM imipramine or 10 μM SR33557 (Fig. 2A). Acid sphingomyelinase inhibition correlated with a dose-dependent inhibition of *N. gonorrhoeae* N309 uptake (Fig. 2B). Compared to untreated control

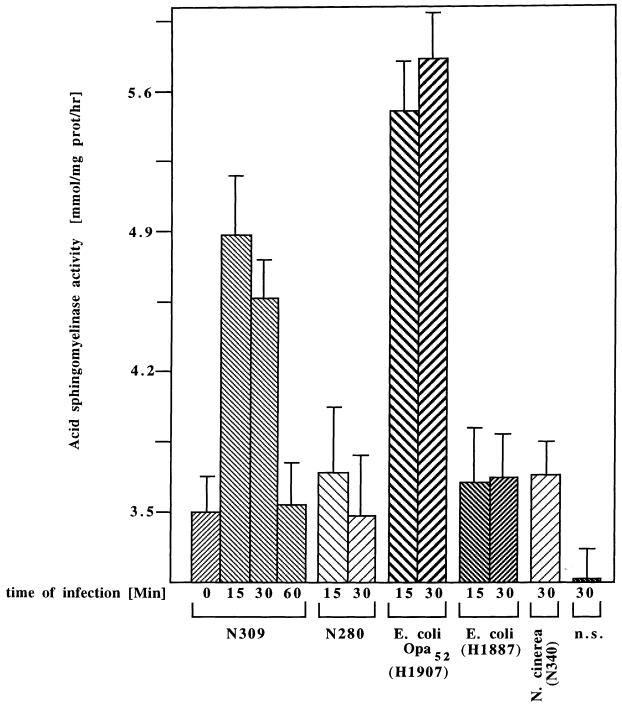


Fig. 1. Infection of human phagocytes with Opa_{52}-expressing *N. gonorrhoeae* activates the acid sphingomyelinase. Human phagocytic cells were infected with the Opa_{52}-positive *N. gonorrhoeae* N309, the Opa-negative N280, non-pathogenic *N. cinerea* N340 as well as *E. coli* transfected with an expression vector for Opa_{52} or a control vector for the indicated time. After infection, cells were lysed, the acid sphingomyelinase was immunoprecipitated and the activity of the enzyme was determined by consumption of [^{14}C]sphingomyelin. Activity is expressed as mmol sphingomyelin consumption/mg (total protein) h. The non-specific immunoprecipitation (n.s.) was performed with an irrelevant antibody after infection of phagocytic cells with N309 for 30 min and contained no significant sphingomyelinase activity. The results indicate a specific activation of the acid sphingomyelinase by the Opa expressing bacteria, whereas *N. gonorrhoeae* or *E. coli* lacking Opa proteins fail to stimulate the acid sphingomyelinase. The bars represent the means ± standard deviation of three independent experiments with duplicate samples.

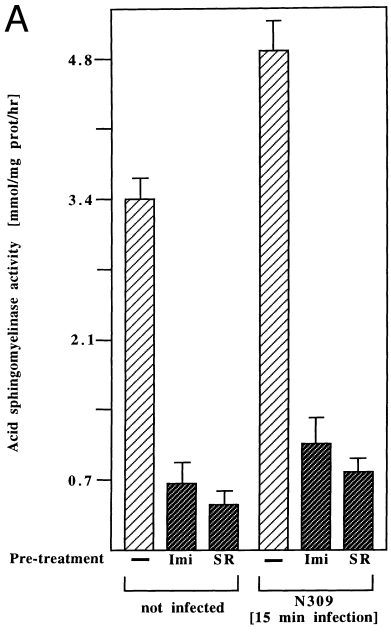


Fig. 2. Inhibition of the acid sphingomyelinase prevents *N. gonorrhoeae* invasion into, but not adhesion to human phagocytes. A: Incubation of human phagocytes with imipramine for 15 min or SR33557 for 1 min results in inhibition of the acid sphingomyelinase. The cells were pre-treated for 15 min with 50 μM imipramine (Imi), for 1 min with 10 μM SR33557 (SR) or solvent only (−), infected with N309 for 15 min and the activity of the acid sphingomyelinase in uninfected or infected cells was determined as described in Fig. 1. Bars represent the means ± standard deviation of two experiments performed in duplicate. B: The inhibition of the acid sphingomyelinase activity by imipramine or SR33557 results in a dose-dependent inhibition of *N. gonorrhoeae* N309 internalization into human phagocytic cells. JOSK-M cells were pre-treated with the indicated concentrations of imipramine or SR33557 and infected with N309. The percentage of infected cells was determined by co-staining of bacteria and the phagosomal membrane marker h-lamp-2. Bars indicate the means ± standard deviation of three independent experiments. C: The block of bacterial internalization upon inhibition of the acid sphingomyelinase is not due to an altered adhesion of the bacteria to the cells as revealed by confocal microscopy experiments. The samples were simultaneously stained with antibodies directed against *N. gonorrhoeae* (middle row, green in the overlay) and antibodies recognizing human lysosome associated membrane protein 2 (h-lamp-2) (left row, red in the overlay). The overlays show that under standard conditions Opa_{52}-expressing *N. gonorrhoeae* are taken up efficiently as evidenced by co-localization with the intracellular marker h-lamp-2. In contrast, in cells pretreated with imipramine *N. gonorrhoeae* still bind to the phagocytes, but fail to be internalized. D: Addition of 50 nM C_{16} ceramide 5 min prior to infection of cells treated with imipramine or SR33557, restores internalization of *N. gonorrhoeae* N309 into those cells. JOSK-M cells were treated for 15 min with 50 μM imipramine or for 1 min with 10 μM SR33557 or left untreated. C_{16} ceramide was added 10 min after addition of imipramine as indicated or 1 min after SR33557 and after further 5 min incubation the infection was initiated as above. Cells were infected for 15 min, fixed, crystal violet-stained and internalization of the bacteria was determined by analyzing at least 100 cells using a Nikon Optiphot microscope. Shown are the means ± standard deviation of two independent experiments.

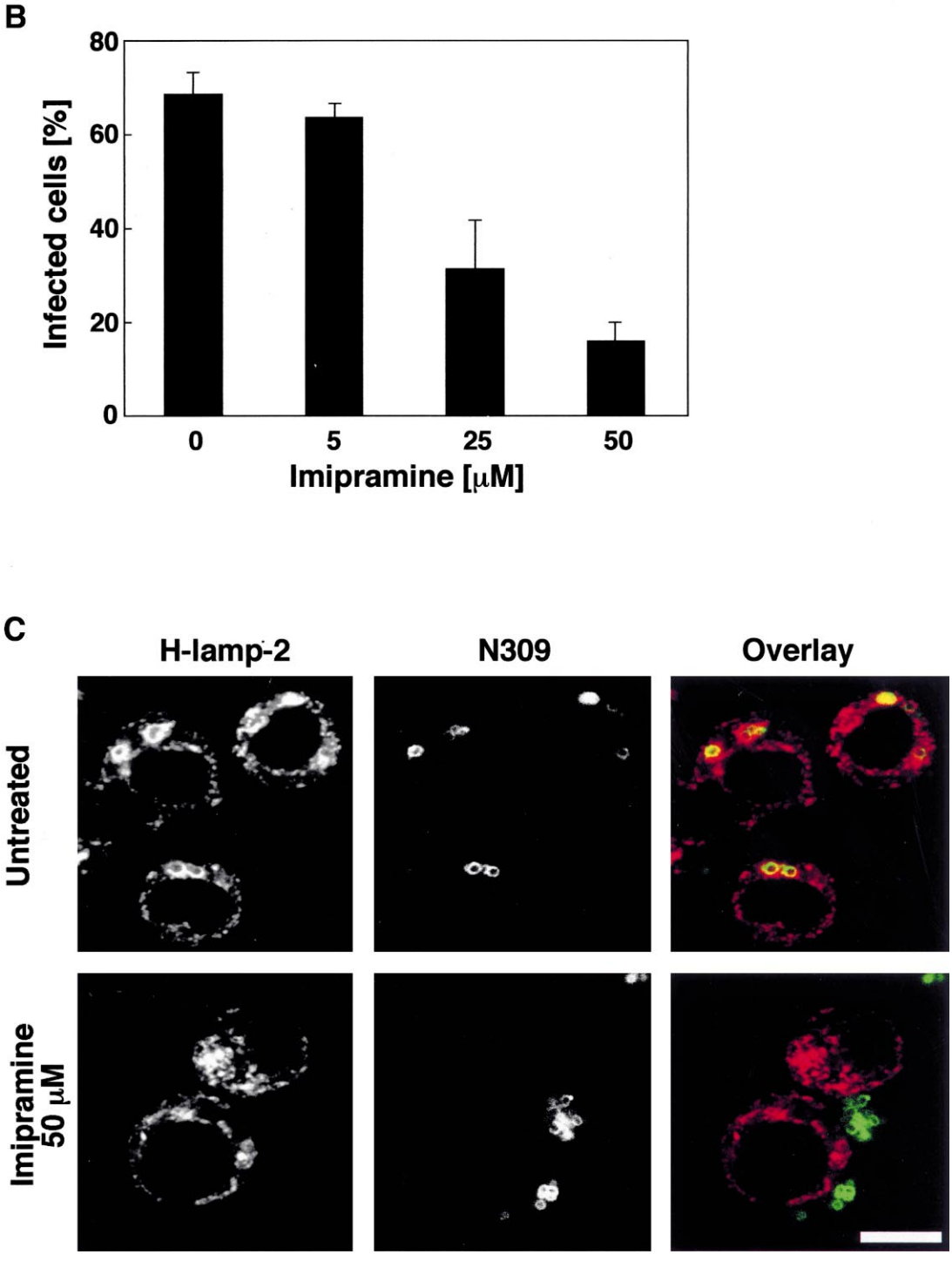
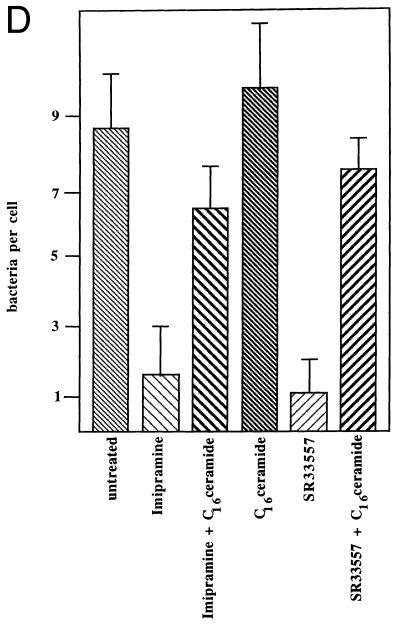


Fig. 2 (*continued*).

cells there was a more than 4–5 fold reduction of bacterial internalization in cells pre-incubated with imipramine or SR33557. Interestingly, cells treated with imipramine or SR33557 still bound Opa_{52}-expressing gonococci, but failed to internalize the bacteria, whereas control cells took up the pathogens into an intracellular vesicular compartment characterized by h-lamp-2 molecules (Fig. 2C and not shown).

In order to exclude that imipramine or SR33557 blocked *N. gonorrhoeae* internalization by inhibition of other molecules than acid sphingomyelinase, we added 50 nM C_{16} ceramide to imipramine or SR33557-treated or untreated cells 5 min prior to infection with *N. gonorrhoeae* N309 (Fig. 2D). Experiments employing [$^{14}C_{16}$]ceramide (sphingoid base: sphingosine) indicate that approximately 5% of the ceramide is incorporated into the cells and not further metabolized during the short, 5-min incubation time (not shown). Since acid sphingomyelinase metabolizes sphingomyelin to ceramide, the addition of C_{16} ceramide should specifically restore the function of acid sphingomyelinase in imipramine or SR33557-treated cells without reconstitution of other enzymes potentially targeted by imi-



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pramine or SR33557. The results of these studies reveal that addition of acid sphingomyelinase to imipramine-treated cells completely restores internalization of *N. gonorrhoeae* correlating with a significant ASM activity in those cells (Fig. 2D).

Taken together, these results suggest a crucial role for the acid sphingomyelinase in CEACAM receptor-mediated phagocytosis by human phagocytic cells.

Next, we tested the consequence of acid sphingomyelinase inhibition for other signaling molecules during the uptake process. In particular, we tested whether the acid sphingomyelinase might be involved in the activation of the Src-like tyrosine kinases Hck and Fgr upon internalization of Opa_{52}-expressing *N. gonorrhoeae* N309. These kinases have been previously shown to be activated by and required for the CEACAM receptor-mediated opsonin-independent phagocytosis of *N. gonorrhoeae* by human phagocytic cells [10]. To this end, we determined the activity of Src-like tyrosine kinases upon infection with *N. gonorrhoeae* N309 in cells pretreated with imipramine or left untreated (Fig. 3A). The results demonstrate that the acid sphingomyelinase inhibitor imipramine almost completely prevented the activation of the Src-like tyrosine kinases Hck and Fgr upon infection of JOSK-M cells with *N. gonorrhoeae* N309 (Fig. 3A). In addition, we investigated if the inhibition of acid sphingomyelinase by imipramine affects additional signalling events stimulated during CEACAM receptor-mediated uptake of Opa_{52}-expressing *N. gonorrhoeae*. Therefore, JOSK-M cells pre-treated or not for 15 min with 50 μM imipramine were infected with *N. gonorrhoeae* N309 or non-pathogenic *N. cinerea* and JNK activity was determined. As shown in Fig. 3B, infection of the phagocytic cells with N309 stimulated a pronounced induction of JNK activity, whereas imipramine pre-treatment abolished the increase in JNK activity.

To further investigate the interaction of the acid sphingomyelinase and protein tyrosine kinases we inhibited Src-like tyrosine kinases using lavendustin and determined the activity of the acid sphingomyelinase in those cells. In contrast to the experiments using imipramine, an inhibition of Src-like tyrosine kinases using lavendustin did not affect the stimulation of the acid sphingomyelinase by *N. gonorrhoeae* N309 (Fig. 3C) suggesting that the acid sphingomyelinase functions upstream of Src-like tyrosine kinases in the internalization process.

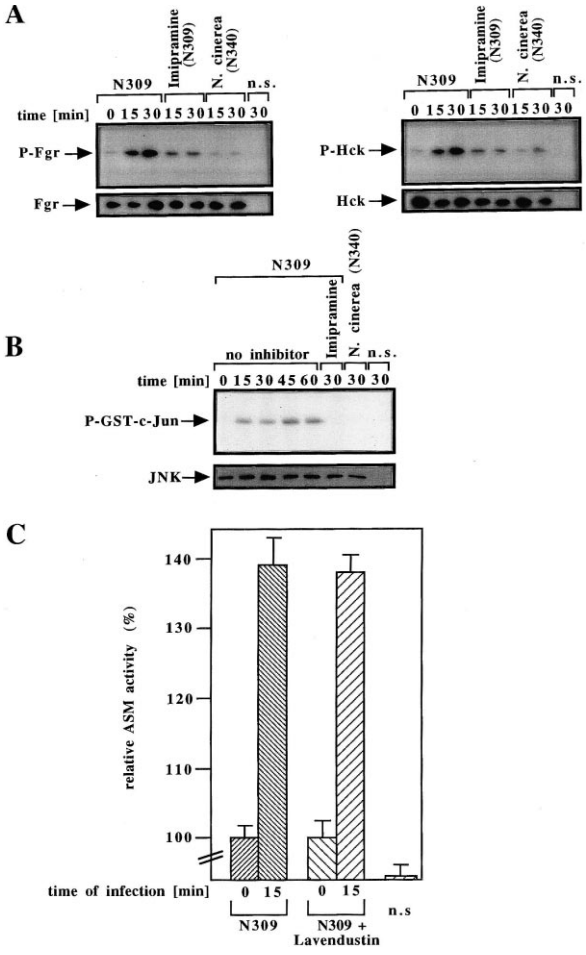

Fig. 3. Inhibition of the acid sphingomyelinase prevents *N. gonorrhoeae* induced activation of Src-like tyrosine kinases and JNK. A,B: Human JOSK-M cells were pretreated for 15 min with 50 μM imipramine or solvent only, infected for the indicated time with *N. gonorrhoeae* N309 or non-pathogenic bacteria, lysed and the Src-like tyrosine kinases Hck and Fgr (A) or JNK (B) were immunoprecipitated. The activity of the kinases was determined by autophosphorylation in the presence of [γ-^{32}P]ATP (A) or by incubation with the substrate GST-c-Jun (B), respectively. Proteins were separated by SDS–PAGE and analyzed by autoradiography. The upper panels display the autophosphorylated kinase (P-Fgr, P-Hck) or the phosphorylated substrate (P-GST-c-Jun). In the lower panels an aliquot of the immunoprecipitates was blotted and probed with specific antibodies to demonstrate similar amounts of immunoprecipitated enzyme in each sample. Immunoprecipitations with non-specific antibodies (n.s.) from JOSK-M cells infected for 30 min with N309 show the specificity of the immunoprecipitations. The results indicate that the activation of Src-like tyrosine kinases as well as JNK upon infection of phagocytes with *N. gonorrhoeae* depends on the function of the acid sphingomyelinase. The blots are representative for three similar experiments. C: Inhibition of Src-like tyrosine kinases using the inhibitor lavendustin (15 min preincubation, 10 μM) does not influence the activation of the acid sphingomyelinase upon infection of human phagocytic JOSK-M cells with *N. gonorrhoeae* N309. The bars represent means ± standard deviation of two experiments with duplicate samples.

4. Discussion

In the present manuscript we provide evidence for an important function of the acid sphingomyelinase in the opsonin-independent, Opa_{52}-mediated internalization of *N. gonorrhoeae* by human phagocytes and, furthermore, in the activation of CEACAM receptor-triggered signalling pathways. Imipramine as well as SR33557 blocked Opa_{52}-mediated internalization. Re-addition of C_{16}-cermide to those cells restored internalization. Since cermide, the only product of ASM activity, restores internalization of *N. gonorrhoeae* in cells treated with the inhibitors, it is very likely that ASM and ceramide and not other proteins are targeted by the two inhibitors mediate internalization.

Imipramine has been shown to induce proteolytic degradation of the acid sphingomyelinase by a still unknown mechanism [14]. SR33557, which is structurally similar to imipramine, acts much faster and without a detectable degradation of p75 or p72 ASM suggesting that this inhibitor might act in a different manner than imipramine. A rapid inhibition of ASM in JOSK-M cells is consistent with previous studies on this inhibitor [15].

Our studies add another model system to the growing number of receptors activating ASM. Those receptors include CD95, TNF, CD5, CD28, or ICAM [4,16–22]. However, it is unknown how these receptors increase the activity of the ASM. Preliminary data from our group show a modification of ASM activity by nucleotides, phosphatidylinositol-phosphates and even tyrosine phosphorylation in vitro, but it is unclear whether these factors or modifications are involved in ASM activation in vivo.

Though the acid sphingomyelinase has been thought to localize selectively to lysosomes, it has been recently demonstrated that this enzyme activity is also present in transport vesicles and can be secreted into the extracellular space [23]. Vesicles containing acid sphingomyelinase may be mobilized by CEACAM receptor engagement, a process which may lead to the presence of sphingomyelinase activity at the sites of bacterial adhesion. There, the acid sphingomyelinase gets access to sphingomyelin, which is predominantly located in the extracellular leaflet of the plasma membrane. Rapid formation of ceramide by sphingomyelinase activity has been shown to result in generation of ceramide-rich membrane microdomains [24]. Glycosphingolipid- and sphingomyelin-enriched membrane domains have been referred to as detergent insoluble glycolipid-enriched complexes (DIGs) or lipid rafts and they seem to influence the distribution of membrane receptors. Especially glycosylphosphatidylinositol (GPI)-anchored proteins and the doubly acylated Src tyrosine kinases have been reported to preferentially partition into DIGs [25,26]. Interestingly, the CEACAM6 receptor, one of the Opa_{52}-binding receptors that are found on phagocytic cells, possesses a GPI-anchor. In addition, the Src family kinases Hck and Fgr are profoundly activated upon CEACAM receptor engagement by Opa_{52}-expressing bacteria. Therefore, it is conceivable that the activation of the acid sphingomyelinase and the generation of ceramide establish specific membrane properties in the vicinity of the bacteria that ensure a favorable microenvironment for CEACAM receptor-Src family kinase crosstalk. In this model, activation of the acid sphingomyelinase would be a prerequisite for efficient signal transmission and bacterial internalization, but the enzyme would not be directly involved in the signalling cascade and rather provide the context for CEACAM receptor-initiated signalling events. Such a function of the acid sphingomyelinase would also explain the fact that the enzyme is not only activated in response to bacterial invasion, but also by a whole variety of receptors as mentioned above. Interestingly, stimulation of acid sphingomyelinase activity by these receptors generally is very fast, pointing to a role for this enzyme in receptor proximal signalling events. Some of these receptors, in particular CD95 and TNF, trigger apoptosis, which has been suggested to be one of the major functions of cellular ceramide, however, the majority of these receptors are co-stimulatory or even prevent apoptosis. Therefore, a simple connection of the acid sphingomyelinase to one particular function such as apoptosis seems to be unlikely and a role in the translocation of receptors into lipid rafts and/or receptor clustering might explain the involvement of the enzyme in multiple signalling pathways.

Importantly, several studies provide evidence that sphingolipids including ceramide are capable of activating Src-like tyrosine kinases directly. In particular, synthetic C6-ceramide has been demonstrated to rapidly activate p56Lck in lymphocytes [27]. Therefore, it might be possible that acid sphingomyelinase-generated ceramide has several distinct functions in the internalization process. First, ceramide could help to establish membrane microdomains that recruit receptors and Src-like tyrosine kinases, and second, ceramide could directly stimulate Src-like tyrosine kinases.

It is interesting to note that not only activation, but also an inhibition of the acid sphingomyelinase can lead to alterations in the composition of membranes. This in turn could impair the ability of the pathogens to adhere to the host cell and therefore explain a reduced internalization upon imipramine treatment. However, our immunofluorescence studies indicate that *N. gonorrhoeae* still adheres to imipramine-treated cells, but is not efficiently internalized. In accordance with these observations, genetic deficiency of the acid sphingomyelinase in fibroblasts obtained from a Niemann–Pick-patient Type A almost completely prevents uptake of *N. gonorrhoeae* but does not alter the adhesion of the bacteria to those cells [4].

In contrast to the Opa_{52}-positive *N. gonorrhoeae* N309 and *E. coli* H1907, neither the Opa-negative, pili-expressing MS11 variant N280, nor commensal *N. cinerea* N340 or *E. coli* H1887 induced significant activation of the acid sphingomyelinase upon interaction with human phagocytes. This indicates a specific stimulation of the acid sphingomyelinase by the Opa_{52}-mediated binding to CEACAM receptors. The interaction of bacterial Opa_{52}-protein and CEACAM molecules results in the efficient, opsonin-independent internalization of the bacteria by phagocytic cells [10], but the function of this process in the context of the infection is still elusive. However, since most gonococci recovered from human volunteers experimentally challenged with Opa-negative *N. gonorrhoeae* MS11 expressed Opa_{52} or a homologue [28], it is reasonable to speculate that the expression of Opa_{52} by *N. gonorrhoeae* provides a selective survival advantage in vivo. Since the cytoplasmic domain of CEACAM1 contains an ITIM motif known to associate with the tyrosine phosphatase SHP-1 and, thus, to mediate inhibitory signals, the engagement of CEACAM receptors by *N. gonorrhoeae* may result in a suppression of certain neutrophil functions [29]. This notion is supported by the result that overexpression of CEACAM receptors is

able to suppress tumor growth in a rodent colon carcinoma model [30] and it has been speculated that the recruitment of SHP-1 to the CEACAM receptor is responsible for this effect. However, since SHP-1 activity is downregulated upon Opa_{52}-mediated stimulation of CEACAM receptors in human phagocytes, this phosphatase does not seem to contribute to a negative signaling event in these cells [11]. Further, in both epithelial and phagocytic cells internalization of the pathogenic gonococci is accompanied by increased JNK activity [4,10]. Since JNK has been implicated in signaling pathways in response to stress that impinge on stress-induced cellular apoptosis [31], the activation of this pathway may also provide a survival advantage for the bacteria.

Finally, it has been recently demonstrated that a gonococcal outer membrane protein, PorB, translocates into membranes of eukaryotic cells and interferes with normal phagosome maturation in primary human phagocytes [32]. Intimate attachment of Opa_{52}-expressing bacteria to CEACAM receptor-rich membrane microdomains may enable the translocation of PorB into host cell membranes and prevent maturation of the newly formed phagosomes to mature lysosomes. In addition, CEACAM receptor-mediated uptake may target the pathogen to a different intracellular compartment than bacteria phagocytosed via opsonin-dependent mechanisms. This may permit the intracellular bacteria to survive for prolonged times. Future studies on the intracellular accommodation of Opa_{52}-expressing bacteria following CEACAM receptor-mediated internalization should help in elucidating the intracellular survival strategy of these pathogens.

Acknowledgements: The authors thank Dr. T. Rudel and Prof. Dr. K. Sandhoff for valuable reagents as well as Caroline Müller for technical assistance. C.R.H. acknowledges the support of the Tübinger Stipendienstiftung and of the Stifterverband für die Deutsche Wissenschaft. The work was supported by DFG Grant Gu 335/10-1 to E.G. and Fe 390/3-1 to K.F. and by grants of the Fonds der Chemischen Industrie to T.F.M.

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